CYTOTYPIC VARIATION IN *PHLOX PILOSA* SSP. *PILOSA* (POLEMONIACEAE) AT THE WESTERN EDGE OF ITS RANGE IN THE CENTRAL UNITED STATES

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ABSTRACT

Polyploidy occurs frequently in plants, and some species exhibit intraspecific variation in ploidy level, or cytotypic variation. This study investigated cytotypic variation in *Phlox pilosa* L. ssp. *pilosa* (Polemoniaceae) along the western edge of its range from north central Texas to southeastern Kansas. Flow cytometry was used to assess genome size for individuals from 46 populations, and meiosis was observed for several populations using compound light microscopy to enable linkage of flow cytometry data with chromosome number. Results demonstrate that *P. pilosa* ssp. *pilosa* occurs as diploid and tetraploid populations in the region, with tetraploids generally occurring to the west of diploid populations. Three populations exhibited genome size values that were higher than expected for tetraploids, possibly due to presence of B chromosomes. This work contributes to an improved understanding of polyploidy in the genus *Phlox*.

RESUMEN

La poliploidía ocurre con frecuencia en las plantas, y algunas especies presentan variación intraespecífica en su nivel de ploidía, o variación citotípica. Este estudio investigó la variación citotípica en *Phlox pilosa* L. ssp. *pilosa* (Polemoniaceae) a lo largo del borde occidental de su área de distribución desde el centro norte de Texas hasta el sureste de Kansas. Se utilizó la citometría de flujo para evaluar el tamaño del genoma de individuos de 46 poblaciones, y se observaron meiosis en varias poblaciones utilizando microscopía óptica para permitir la vinculación de los datos de citometría de flujo con el número de cromosomas. Los resultados demuestran que *P. pilosa* ssp. *pilosa* ocurre como poblaciones diploides y tetraploides en la región, con las tetraploides generalmente al oeste de las poblaciones diploides. Tres poblaciones mostraron valores del tamaño del genoma que eran más altos de lo esperado para tetraploides, posiblemente debido a la presencia de cromosomas B. Este trabajo contribuye a una mejor comprensión de la poliploidía en el género *Phlox*.

KEY WORDS: autopolyploidy, chromosome count, cytotype, flow cytometry, *Phlox*, Polemoniaceae, polyploidy

INTRODUCTION

Polyploidy is frequent in plants and is considered an important factor in plant evolution (Otto & Whitton 2000; Adams & Wendel 2005; Soltis et al. 2009; Soltis et al. 2010). Autopolyploidy, or genome doubling within a species, can lead to intraspecific ploidy level (cytotypic) variation (although some autopolyploids alternatively meet criteria for species recognition; see Soltis et al. 2007). Such cytotypic variation within species may be underappreciated, as systematists have sometimes made assumptions about ploidy levels for entire taxa based on one or few chromosome counts. Advances in flow cytometry methods enable rapid assessment of genome size and inference of ploidy level (Doležel & Bartoš 2005), and recent studies have demonstrated intriguing cytotypic variation in some species (e.g., Balao et al. 2009; Cires et al. 2009; Whittemore & Olsen 2011). Documentation of cytotypic variation is a critical first step toward understanding ecological, genetic, and taxonomic consequences of autopolyploidy in particular groups.

Phlox pilosa L. (Polemoniaceae) is a showy, upright, perennial species ranging throughout most of the eastern United States, from the east coast to the edge of the Great Plains and Central Texas (Wherry 1955; Great Plains Flora Association 1986; Gleason & Cronquist 1991). *Phlox pilosa* exhibits noteworthy morphological variation across its range, and several subspecies are usually recognized (Wherry 1955; Levin & Smith 1965; Levin 1966; Locklear 2011; see also Ferguson 1998). *Phlox pilosa* ssp. *pilosa* occurs in prairies and woodland openings across most of the general range of *P. pilosa* as a whole, with westernmost populations occurring in north central Texas and south central Oklahoma. Most published chromosome counts for *P. pilosa* are diploid, and represent the wide-ranging *P. pilosa* ssp. *pilosa* (e.g., Kelly & Wahl 1928; Flory 1931 [*P. argillacea* Clute &

Ferris, a taxonomic synonym], 1934; Meyer 1944; Levin & Smith 1965; Levin 1966, 1968; Smith & Levin 1967; see also Levin & Schaal 1970; Levy & Levin 1974). However, there has long been a suggestion of polyploidy near the western edge of the range of *P. pilosa* ssp. *pilosa*. Smith and Levin (1967) reported a tetraploid chromosome count for material of *P. pilosa* ssp. *pilosa* from a mile south of Sachse, Texas (Dallas County, in north central Texas) and one tetraploid count for material of *P. pilosa* ssp. *pilosa* from the state of Arkansas (precise locality unknown). This has led to some question regarding ploidy level and possible cytotypic variation in *P. pilosa* ssp. *pilosa* in the region.

Phlox pilosa is one of the most thoroughly studied species of the genus *Phlox* (due particularly to the extensive systematic and ecological research conducted by D.A. Levin and colleagues in the 1960s and 1970s; e.g., citations listed above), and further work on *P. pilosa* advances the utility of *Phlox* as a study system. *Phlox* is a genus of ca. 60 species of perennial herbs occurring mostly in North America (Wherry 1955; Locklear 2011; Ferguson et al. in prep. [FNA vol. 15]). The base chromosome number for *Phlox* is x=7 (Flory 1934), and diploid, tetraploid and hexaploid chromosome counts have been reported (see Kelly & Wahl 1928; Flory 1931, 1934, 1937, 1948; Meyer 1944; Levin 1964, 1966, 1968; Levin & Smith 1965; Eater 1967; Smith & Levin 1967; Löve 1971; Strakosh 2004; Fehlberg & Ferguson 2012 and in press). Furthermore, polyploidy has been implicated as a factor contributing to complicated patterns of phylogeny, including incongruence between nrDNA and cpDNA phylogenies for samples of *P. pilosa* and close relatives (Ferguson & Jansen 2002; see also Ferguson et al. 1999). Study of cytotypic variation within *P. pilosa* ssp. *pilosa* can thus be placed within an extensive broader research context.

In this study, patterns of cytotypic variation in *P. pilosa* ssp. *pilosa* were explored at the western edge of its range, from north central Texas to eastern Oklahoma, western Arkansas, and southeastern Kansas. Flow cytometry was conducted chiefly on fresh field-collected leaf material, as well as previously preserved material for some populations (silica gel-dried, frozen, or herbarium material). Meiotic chromosome counts were performed on bud material from several populations to enable ploidy level inferences based on genome size (Doležel et al. 2007).

MATERIALS AND METHODS

Field collection.—Material was collected from over 50 populations of *P. pilosa* ssp. *pilosa* ranging from the general area of the earlier reported tetraploid count in Texas (Smith & Levin 1967) and north along the western edge of the taxon range as far as southeastern Kansas. Fresh leaf material from an individual plant from each population was reserved for flow cytometry. For some populations, leaves from multiple individuals were collected in silica gel to enable later testing for cytotypic variation within populations. For populations at an early stage of flowering, bud material for chromosome counts was collected (and preserved in a solution of 3 parts 95% ethanol: 1 part glacial acetic acid, with subsequent transfer to 70% ethanol). Voucher specimens from all populations were prepared and deposited in the Kansas State University Herbarium (KSC). This study was further augmented with material from several populations previously collected (material that had been collected in silica gel or fresh-frozen and stored at -70°C, and one recent herbarium specimen).

Flow cytometry.—DNA content per cell was assessed for individuals using flow cytometry. At least one individual was sampled per population, with additional within-population sampling for 11 populations. Leaf material (0.04–0.10 grams) was chopped with a fresh razor blade in 2 mL of chopping buffer specified by Davison et al. (2007; modified from Bino et al. 1993). The resulting slurry was filtered through 30 µm nylon mesh (Small Parts, Inc.), followed by vortexing and centrifugation (500 RCF for 7 minutes). The supernatant was decanted off of the pellet, or if a pellet did not form, approximately half of the liquid was decanted. Propidium iodide staining solution (BioSure; 700 µL) was added to each sample to fluorescently stain the DNA; and 2 µL of chicken erythrocyte nuclei (CEN) singlets (BioSure) were added to each sample as an internal standard. Samples were vortexed and stored on ice for 1–2 hours prior to processing on a Becton Dickinson FACS-Calibur flow cytometer at the Flow Cytometry Lab of the College of Veterinary Medicine, Kansas State University.

The flow cytometer quantifies the amount of DNA by measuring the fluorescence per nucleus. The result-

ing histogram for each sample was analyzed using CellQuest software (Becton Dickinson), and a coefficient of variation (CV) was obtained. To ensure highest quality data, we aimed to retain samples with measurements based on ca. 10,000 nuclei per sample and having CV values <5% (see Galbraith et al. 1997; Doležel & Bartoš 2005). Picogram (pg) amounts were then calculated using the equation from Doležel and Bartoš (2005).

Chromosome counts.—Chromosome counts were conducted using a modified version of B.L. Turner's squash technique for meiotic cells (Jones & Luchsinger 1986). These counts enabled linking of picogram DNA amounts to chromosome numbers for *P. pilosa* ssp. *pilosa*, and thus inference of ploidy levels based on flow cytometry data.

RESULTS

Flow cytometry data were retained for 46 populations: these samples had 10,005–11,205 nuclei scored and CV values from 1.91–4.98% (Table 1). Flow cytometry worked well for the *Phlox* material; most runs yielded high quality data, although some samples did not meet criteria for inclusion (see Materials and Methods; due to lower numbers of events, high CV values, or, occasionally, poorly defined peaks). Based on these data (Table 1), cytotypic variation in *P. pilosa* ssp. *pilosa* was inferred, with 13 diploid populations, 30 tetraploid populations, and three unusual populations with higher DNA content (discussed below). DNA content per nucleus for the diploid and tetraploid populations averaged 10.66 pg (range 9.22–13.36 pg) and 21.90 pg (range 18.27–24.81 pg), respectively (based on measurements of all individuals; Table 1). The three remaining populations had an average DNA content per nucleus of 26.55 pg (range 23.59–29.46 pg; Table 1).

For 11 populations, additional individuals (2–9) were analyzed. No cases of intrapopulational cytotypic variation were inferred (Table 1). However, measured within population variation in picogram values ranged from 0.26 pg (in diploid population 16) to 4.13 pg (in tetraploid population 17; Table 1).

Chromosome counts were obtained, including a diploid count (n=7) and a tetraploid count (n=14; Fig. 1a, 1b; Table 1). Cells undergoing meiosis were also observed for two of the three populations that exhibited high genome size values (populations 12 and 13). Cells from some buds of population 12 showed clear tetraploidy (n=14), while those from other buds suggested additional chromosomes or chromosomal fragments (although clear counts could not be obtained). Cells from buds of population 13 appeared to have more chromosomes than the tetraploid material (n=17+), although the chromosomes could not be sufficiently spread apart. While it is possible that higher level polyploids are present, these results may also be due to the presence of B chromosomes (supernumary chromosomes; see Smith & Levin 1967 for reports of B chromosomes in *P. pilosa*; see also Meyer 1944 for notation of "fragments" in meiotic figures of some *Phlox* taxa).

Mapping the cytotypic data for sampled populations revealed a generally east-west pattern (Fig. 2), with diploid populations occurring in the eastern part of the sampling range, tetraploid populations in the western part, and the three populations with unusually high DNA content in southeastern Oklahoma (Fig. 2).

DISCUSSION

This study demonstrates that *P. pilosa* ssp. *pilosa* occurs as diploid and tetraploid populations at the western edge of its range from the southeastern Great Plains (sensu Great Plains Flora Association 1986) and south into northern Texas. This variation does not correspond to any previously recognized infraspecific taxa in *P. pilosa*, and no morphological differences were noticed among cytotypes (future work will investigate micromorphology of these populations). Interestingly, two subspecies of *P. pilosa* occurring further west in Texas (onto the Edwards Plateau) are known to be tetraploid (*P. pilosa* ssp. *latisepala* Wherry and *P. pilosa* ssp. *riparia* Wherry; see Levin 1966, 1968; Smith & Levin 1967). Some workers have recognized these Central Texas taxa at the specific level, partly due to the polyploid condition: *P. aspera* E.E. Nelson and *P. villosissima* (A. Gray) Whitehouse (Levin 1968; Levin & Schaal 1970; Levy 1973); or, as a single species under *P. villosissima* (Turner 1998; Locklear 2011). Our documentation of tetraploid populations of *P. pilosa* ssp. *pilosa* in northern Texas (and northward) indicates that there are no ploidy level differences precluding intergradation with the Central Texas taxa. Investigation of population level morphological, genetic, and cytotypic variation are warranted

TABLE 1. Samples of *P. pilosa* ssp. *pilosa*: population number, locality, voucher information, sample material, average DNA content per nucleus based on flow cytometry (n; min.-max. values), and inferred ploidy level based on DNA content (and linked to chromosome count information in addition, as indicated by an asterisk).

Popn.	Locality	Voucher(s)[1]	Sample material	DNA content (pg)	Ploidy level[1, 2]
1	Scott Co., AR	CJF 448	Frozen	12.34 (1)	2x, n=7
2	Sevier Co., AR	CJF 457	Frozen	10.11 (1)	2x, n=7
3	Bourbon Co., KS	LW 26	Fresh	9.56 (1)	2x, n=7
4	Montgomery Co., KS	LW 2	Fresh	22.62 (1)	4x, n=14
5	Neosho Co., KS	LW 24	Fresh	10.50 (1)	2x, n=7
6	Neosho Co., KS	LW 25	Fresh, Silica gel	9.96 (6; 9.22–10.95)	2x, n=7
7	Wilson Co., KS	LW 1	Fresh	22.33 (1)	4x, n=14
8	Carter Co., OK	MHM 3847	Fresh	23.80 (1)	4x, n=14
9	Cherokee Co., OK	LW 19	Fresh	11.27 (1)	2x, n=7
10	Cherokee Co., OK	LW 20	Fresh, Silica gel	20.00 (4; 19.29–21.27)	4x, n=14
11	Cherokee Co., OK	LW 103	Fresh	9.52 (1)	2x, n=7
12	Choctaw Co., OK	LW 107*	Silica gel	25.42 (3; 23.59–26.52)	4x+*, n=14+
13	Choctaw Co., OK	MHM 3854, LW 89*	Fresh, Herbarium	27.87 (2; 26.27–29.46)	4x+*, n=14+
14	Craig Co., OK	LW 22	Fresh	18.27 (1)	4x, n=14
15	Creek Co., OK	LW 5	Fresh	22.99 (1)	4x, n=14
16	Delaware Co., OK	LW 104	Fresh, Silica gel	10.04 (2; 9.91–10.17)	2x, n=7
17	Haskell Co., OK	LW 100	Fresh, Silica gel	21.30 (9; 19.79–23.92)	4x, n=14
18	Latimer Co., OK	LW 15	Fresh	12.04 (1)	2x, n=7
19	Latimer Co., OK	LW 99	Fresh, Silica gel	11.01 (1)	2x, n=7
20	Le Flore Co., OK	CJF 473	Silica gel	13.36 (1)	2x, n=7
21	Mayes Co., OK	LW 21	Fresh	23.01 (1)	4x, n=14
22	McCurtain Co., OK	CJF 470	Silica gel	27.28 (1)	4x+, n=14+
23	Muskogee Co., OK	LW 8	Fresh	22.13 (1)	4x, n=14
24	Muskogee Co., OK	LW 9	Fresh	24.75 (1)	4x, n=14
25	Muskogee Co., OK	LW 10	Fresh	21.00 (1)	4x, n=14
26	Muskogee Co., OK	LW 11, LW 97*	Fresh, Silica gel	20.40 (4; 19.65–20.75)	4x*, n=14
27	Nowata Co., OK	LW 3	Fresh	22.39 (1)	4x, n=14
28	Nowata Co., OK	LW 4	Fresh	22.46 (1)	4x, n=14
29	Okmulgee Co., OK	LW 7	Fresh	24.45 (1)	4x, n=14
30	Okmulgee Co., OK	LW 98	Fresh, Silica gel	22.15 (8; 20.60–23.49)	4x, n=14
31	Pontotoc Co., OK	MHM 3856	Fresh	23.59 (1)	4x, n=14
32	Pushmataha Co., OK	LW 13	Silica gel	23.03 (2; 21.27–24.29)	4x, n=14
33	Pushmataha Co., OK	LW 14	Silica gel	22.54 (1)	4x, n=14
34	Sequoyah Co., OK	LW 17, LW 93*	Fresh	11.75 (1)	2x*, n=7
35	Sequoyah Co., OK	LW 18	Fresh	11.17 (1)	2x, n=7
36	Tulsa Co., OK	LW 6	Fresh	23.97 (1)	4x, n=14
37	Wagoner Co., OK	LW 101	Fresh, Silica gel	24.15 (2; 23.48–24.81)	4x, n=14
38	Wagoner Co., OK	LW 102	Fresh, Silica gel	21.60 (1)	4x, n=14
39	Bowie Co., TX	CJF 418	Frozen	24.79 (1)	4x, n=14
40	Grayson Co., TX	MHM 3848	Fresh	20.06 (1)	4x, n=14
41	Grayson Co., TX	MHM 3849	Fresh	20.95 (1)	4x, n=14
42	Grayson Co., TX	MHM 3850	Fresh	22.53 (1)	4x, n=14
43	Lamar Co., TX	CJF 416, MHM 3851	Fresh, Frozen	20.86 (6; 19.15–22.90)	4x, n=14
44	Lamar Co., TX	MHM 3852	Fresh	22.81 (1)	4x, n=14
45	Lamar Co., TX	MHM 3853	Fresh	24.44 (1)	4x, n=14
46	Red River Co., TX	CJF 417	Frozen	22.89 (1)	4x, n=14

[1]Asterisks indicate chromosome count information: an asterisk in the voucher column signifies the voucher linked to bud material for a chromosome count, and an asterisk in the ploidy level column indicates confirmation of the ploidy level inference (2x or 4x; or 4x and/or higher [4x+]; see text) by the chromosome count.

[2]Diploid, 2x, 9.22–13.36 pg; tetraploid, 4x, 18.27–24.81 pg; populations including individuals with higher than expected DNA content for tetraploids, 4x+, 23.59–29.46 pg (see text).

across the Central Texas taxa and *P. pilosa* ssp. *pilosa* to better understand diversity in the region. Intriguingly, the narrowly endemic *P. pilosa* ssp. *longipilosa* (Waterf.) J. Locklear, occurring in granite outcrops of southwestern Oklahoma, is diploid (MHM & CJF, unpubl. chromosome count data). To our knowledge, polyploidy

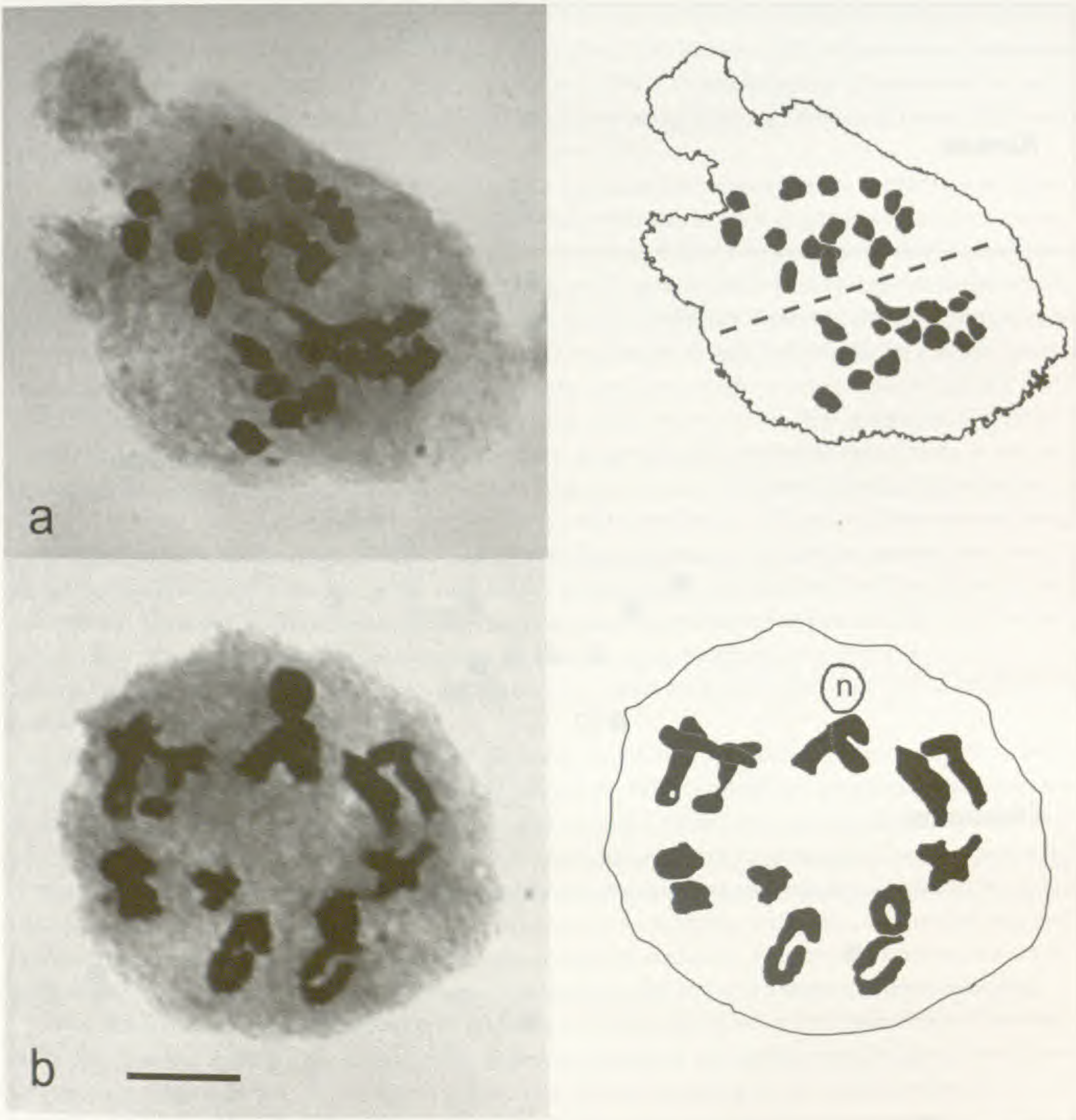

FIG. 1. Images of meiosis from compound light microscopy (with drawings showing interpretation) documenting tetraploidy (n=14) for population 26; **a)** Anaphase I (image, left; and drawing, right); **b)** Metaphase I (image, left; and drawing, right). The scale bar (lower left) indicates 10 µm, and the label "n" (1b, drawing) indicates the nucleolus.

within *P. pilosa* thus occurs in the region sampled in the present study (southeastern Kansas and south into northern Texas; in *P. pilosa* ssp. *pilosa*), west and south through Central Texas (in *P. pilosa* ssp. *latisepala* and *P. pilosa* ssp. *riparia*).

While most populations of *P. pilosa* ssp. *pilosa* examined in this study were inferred to be diploid or tetraploid, three populations in southeastern Oklahoma had unusually high DNA content per nucleus, and compound light microscopy of meiosing cells suggested presence of chromosomes (or chromosome fragments) beyond the tetraploid number. Further cytological study of these populations will be necessary to definitively document ploidy. Accessory, B chromosomes have been documented in *P. pilosa* ssp. *pilosa* (Smith & Levin 1967; in populations from central and northern Alabama, northeastern Arkansas, and northwestern Indiana), and may well explain the variation detected here. Although perhaps less likely, higher ploidy levels are also

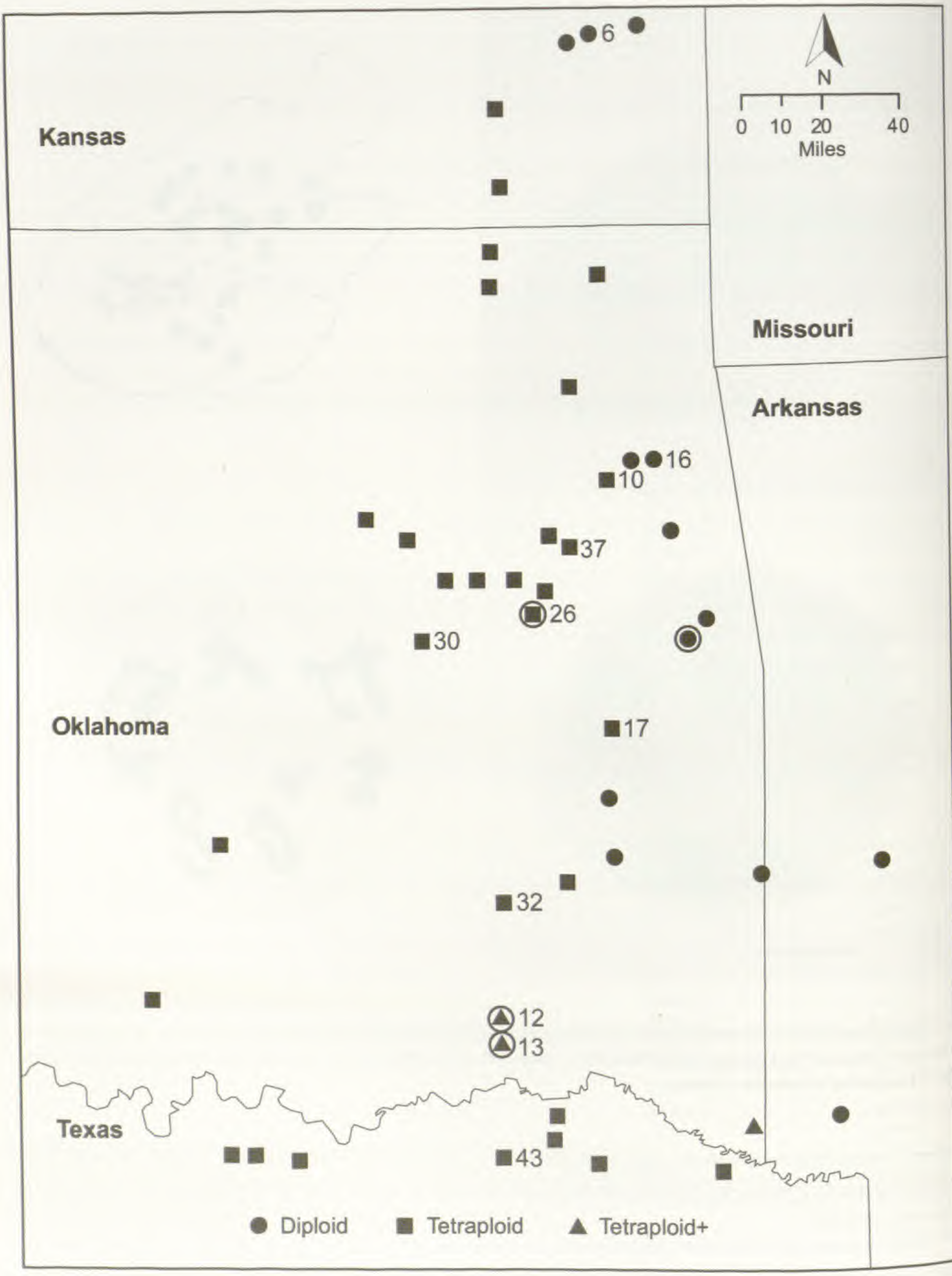

FIG. 2. Map showing localities of *P. pilosa* ssp. *pilosa* samples included, with state names labeled. Diploid populations are indicated with circles, tetraploid populations with squares, and populations with unusually high DNA content ("tetraploid+") with triangles. A circle surrounding a shape symbol indicates that meiosis was observed with compound light microscopy (Table 1). Populations for which more than one individual was analyzed using flow cytometry are indicated by population number (Table 1) to the direct right of the symbol.

known in *Phlox*. For example, Fehlberg & Ferguson (2012; in press) have found diploid, tetraploid and hexaploid cytotypes in *P. amabilis* Brand and *P. woodhousei* (A. Gray) E.E. Nelson (with evidence from chromosome counts, flow cytometry, and population genetic data); Eater (1967) obtained diploid, tetraploid and hexaploid chromosome counts for *P. nana* Nutt. (following current taxon recognition; e.g., Wilken & Porter 2005); and a hexaploid count is known for *P. andicola* E.E. Nelson (Löve 1971).

Tetraploid individuals of *P. pilosa* ssp. *pilosa* have a genome size approximately double that of diploids (averaging 21.90 pg [*n*=58] vs. 10.66 pg [*n*=19]; populations with unusually high genome size values averaged 26.55 pg [*n*=6]). Ranges in genome size within a ploidy level (e.g., 9.22–13.36 pg for individual diploids) were somewhat greater than expected. For example, Fehlberg and Ferguson (2012) found picogram ranges within a ploidy level of less than three picograms for *P. amabilis* and *P. woodhousei*. However, their sampling on a per cytotype basis within a taxon was more limited (with a maximum of eight individuals for a single cytotype [tetraploids] within *P. amabilis* and a maximum of 18 individuals for a single cytotype [also tetraploids] within *P. woodhousei*; Fehlberg & Ferguson 2012). In the present study, there were no clear geographical patterns to genome size variation within cytotypes: in some cases, geographically proximate populations of the same ploidy level differed greatly (e.g., DNA content for two Wagoner County, Oklahoma, tetraploid populations was 21.60 vs. 24.15 pg), and measurements from individuals within populations differed (with the extreme being a tetraploid population with a range in values slightly over four picograms). Variation in genome size observed within a ploidy level for *P. pilosa* ssp. *pilosa* may reflect, at least in part, an error level for flow cytometry measurements in this study; it may reflect true variation in genome size within cytotypes, and this could be due in part to differential presence of B chromosomes (discussed above; see Smith & Levin 1967); or, cytotypic variation may actually be more complex than inferred here (i.e., some additional cytotypes such as triploids and hexaploids may be present).

Spatial patterns of cytotypic variation in *P. pilosa* ssp. *pilosa* show that tetraploids generally occur west of diploid populations, potentially under more xeric conditions. While diploid and tetraploid populations were detected in close proximity (within eight miles), we did not detect mixed cytotype populations (though, within-population sampling was limited). Cytotypes are somewhat separated by geographic features, particularly river systems: for example, the Neosho River in southeastern Kansas separates sampled diploids to the east and tetraploids to the west. Into central Oklahoma (where most of our sampling was conducted), tetraploid populations tend to occur at slightly lower altitudes relative to diploids and west of major river systems, but detailed study of potential ecological correlates of cytotypic variation in the area will require additional sampling.

Documentation of intraspecfic cytotypic variation in *P. pilosa* ssp. *pilosa* at the western edge of its range is intriguing, and sets the stage for further study exploring ecological and cryptic morphological correlates of this variation. This work advances study of systematics of *Phlox* by adding to our understanding of cytotypic variation. Current work in our lab seeks to synthesize chromosome count and flow cytometry data for *Phlox* in a revised taxonomic context. In a broader context, cytotypic variation may in general be underappreciated in plants: for certain, systematists should not assume inference of ploidy level for an entire taxon based on one or few chromosome counts alone. Broader surveys (made easier through techniques such as flow cytometry) and detailed studies of cytotypic patterns promise to improve our general understanding of this aspect of biodiversity in plants.

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